Immunity, Vol. 21, 655–667, November, 2004, Copyright ©2004 by Cell Press

Induction of Secondary and Tertiary Lymphoid Structures in the Skin

Tom Cupedo, Wendy Jansen, Georg Kraal, and Reina E. Mebius*
Department of Molecular Cell Biology and Immunology
VU Medical Center
P.O. Box 7057
1007 MB Amsterdam
The Netherlands

Summary

During embryogenesis a developmental program leading to the formation of lymph nodes and Peyer's patches is initiated. We now show that lymph node-like structures as well as tertiary lymphoid structures can ectopically be induced by intradermal injection of newborn lymph node-derived cells. ICAM-1/VCAM-1-expressing stromal organizers, follicular dendritic cells, lymphatic endothelium, and HEVs in these structures are of donor origin, while all hematopoietic cells are host derived. Formation depends on lymphotoxin-expressing donor cells, whereas further organization requires lymphotoxin-expressing recipient cells. While induced secondary lymphoid structures develop a normal cellular architecture, the degree of organization in tertiary structures is correlated to the immune activation status of the host. These results indicate that the cellular and molecular requirements for the establishment of lymph nodes and tertiary structures are remarkably similar and that hyperactivated lymphocytes can fulfill the role of lymphoid tissue inducer cells during inflammatory responses.

Introduction

The generation of different gene-targeted mice with disturbed formation or organization of lymph nodes (LNs) and Peyer's patches (PPs) provided novel insights into the molecular interactions underlying lymphoid organogenesis (reviewed in Fu and Chaplin, 1999; Mebius, 2003). A prominent role was attributed to lymphotoxin-β receptor (LTβ-R) triggering by membrane bound lymphotoxin-$\alpha_1\beta_2$ (LT) (Futterer et al., 1998; Rennert et al., 1996, 1998). In mice that lack LNs due to a targeted deletion of the gene encoding LTα, engaging the LTβ-R in vivo by agonistic antibodies is sufficient to induce LN genesis (Rennert et al., 1998). Moreover, transgenic expression of LTα at nonlymphoid sites leads to the formation of LN-like aggregates at the site of transgene expression (Kratz et al., 1996). A population of $LT^+CD45^+CD4^+CD3^-$ cells is capable of inducing the formation of PPs as well as nasal-associated lymphoid tissue (NALT) (Finke et al., 2002; Fukuyama et al., 2002), and a similar role for these cells as inducers of LNs is generally expected (Eberl et al., 2004). At birth, $CD45^+CD4^+CD3^-$ cells express surface LT (Kim et al., 2000; Mebius et al., 1997), which supports the notion that $CD45^+CD4^+CD3^-$ cells initiate LN-organogenesis through ligation of the LTβ-R. This signaling induces stromal cells to produce homeostatic chemokines and express adhesion molecules (Cuff et al., 1998; Dejardin et al., 2002; Honda et al., 2001; Mebius, 2003; Muller and Siebenlist, 2003), leading to the recruitment and retention of circulating hematopoietic cells that will subsequently engage in paracrine interactions with the resident stromal cells (Honda et al., 2001).

A subsequent aspect of LN formation, important for increasing the efficiency of antigen sampling by naive lymphocytes, is the segregation and spatial positioning of lymphocytes into T cell areas and follicular dendritic cell (FDC)-containing B cell follicles (Ansel et al., 2000; Fu et al., 1997; Hashi et al., 2001; Ngo et al., 1999, 2001). The generation of B cell follicles is critically dependent on LTβ-R-mediated production of the homeostatic chemokine CXCL13 by stromal cells and FDCs (Ansel et al., 2000). Additionally, for FDCs to mature both the LTβ-R and the TNF-RI need to be triggered (Endres et al., 1999; Fu et al., 1998).

The formation of lymphocyte-specific microdomains is a biological phenomenon that is also observed during several chronic inflammatory conditions. In diseases like Rheumatoid arthritis, Sjögren's syndrome, and Hashimoto's thyroiditis, the presence of ectopic lymphoid structures might contribute to pathology by enhancing the efficiency of autoantigen presentation and may contribute to the generation of autoreactive lymphocytes (Amft et al., 2001; Armengol et al., 2001; Han et al., 2001; Hjelmstrom, 2001; Salomonsson et al., 2002; Weyand et al., 2001).

A major difficulty in characterizing the essential events in the early development and organization of both LNs and inflammatory aggregates is that these events have to be studied in vivo. During embryonic development, as well as in early postnatal life, LNs are not easily accessible, while the ectopic generation of lymphoid structures invariably requires the presence of persistent stimuli through transgene expression (Fan et al., 2000; Kratz et al., 1996; Luther et al., 2000, 2003; Sacca et al., 1998). In this study, we describe the generation of ectopic LN aggregates in adult mice through a single injection of newborn LN-derived cells. Within these aggregates, lymphatic endothelium, high endothelial venules (HEVs), and stromal cells persist that are of donor origin. When induced in naïve adult mice, there is no apparent organization; however, upon induction in newborn mice, full T/B cell segregation is achieved and FDC-containing B cell follicles are formed, a process dependent on LT expression by recipient cells and mimicking normal lymphoid organ development. Finally, the disorganized aggregates induced in adult mice can be transformed into organized tertiary structures after transient immune activation, showing flexibility of the induced structures, which can open new possibilities for studying the formation and organization of both secondary and tertiary lymphoid organs.

*Correspondence: r.mebius@vumc.nl

Results

Induction of Lymphoid Neoorganogenesis In Vivo

Within the newborn murine LN, stromal cells expressing ICAM-1 and VCAM-1 are abundantly present (Figure 1A). The hematopoietic ($CD45^+$) compartment at this time is still virtually devoid of T cells, but it does contain B cells; however, the major cell population consists of $CD45^+CD4^+CD3^-$ cells (Kim et al., 2000; Mebius et al., 1997), which are the presumed LN-inducer population and cellular source of LT. This prompted us to determine whether their capacity to engage in the complex interactions leading to lymphoid neoorganogenesis could be studied at ectopic sites. Mesenteric LNs (MLNs) were dissected at the day of birth, and single-cell suspensions were made. These suspensions were subsequently injected intradermally in the abdominal skin of adult recipients. After 2 weeks, mice were sacrificed, and the abdomen was inspected for the presence of LN-like aggregates. At this time, macroscopic LN-like structures (Figure 1B), which upon dissection and immunohistochemical analysis revealed the generation of a novel lymphoid aggregate, could be detected. In contrast to the still undeveloped newborn LNs from which the injected cells were isolated, these newly formed structures now contained both T and B lymphocytes, although segregation into separate areas was never observed (Figure 1C). In addition, numerous HEVs were present, and these vessels coexpressed the mucosal addressin MAdCAM-1 and the peripheral addressin PNAd (Figure 1D). $CD11c^+$ dendritic cells (DCs) were also readily detected in the induced aggregates (Figure 1E). Therefore, all components normally seen in LNs could be found in these induced LN-like structures. Noteworthy, ectopic structures could never be induced by using MLNs isolated from adult animals ($n = 5$).

Surprisingly, LN-like structures could also be induced in $LT\alpha^{-/-}$ mice upon intradermal injection of cells derived from C57BL/6 newborn MLNs, and these structures resembled the aggregates seen in C57BL/6 mice. Again, T and B cells were present, although distinct areas were absent (Figure 1F). Furthermore, HEVs expressing both MAdCAM-1 and PNAd were abundantly present (Figure 1G), and many $CD11c^+$ DCs were detected (Figure 1H). These data indicate that the newborn LN contains all the necessary cells to initiate the formation of a novel lymph node-like structure at a nonpredestined site.

Induction of Lymphoid Neogenesis Is Dependent on LTβ-R Signaling

Since induction of neolymphoid structures occurred independent of host-derived LT, we set out to determine the importance of LTβ-R signaling in donor MLNs. C57BL/6 newborn MLNs were compared to rudimentary MLNs (rMLNs) from $LT\alpha^{-/-}$ mice (Figures 2A–2B). Within these rudimentary structures, LTβ-R-expressing stromal cells (Figure 2B) as well as $CD45^+CD4^+CD3^-$ cells were present, albeit that the percentage of $CD45^+CD4^+CD3^-$ cells was reduced (Figure 2A). However, upon intradermal injection, cells from newborn $LT\alpha^{-/-}$ rMLNs did not initiate the formation of lymphoid aggregates ($n = 5$), even after injections of five times more cells then used normally (data not shown). To formally prove that this inability to induce lymphoid aggregates was due to the absence of LTβ-R signaling, an agonistic anti-LTβ-R antibody was coinjected intradermally with newborn $LT\alpha^{-/-}$ rMLN cells. After 2 weeks, lymphoid structures could be dissected that contained lymphatic vessels, dendritic cells, HEV, and some lymphocytes (T and few B lymphocytes) (Figures 2C–2F). The efficiency with which these structures were formed was however markedly lower compared to C57BL/6 MLNs, and formation required more donor cells. These data show that induction of ectopic lymphoid aggregates is critically dependent on LTβ-R signaling, and that enforced LTβ-R signaling in rudimentary MLNs from $LT\alpha^{-/-}$ mice at the day of birth can partly mimic the situation in wild-type mice, in which LTβ-R signaling is initiated before birth (Rennert et al., 1998).

Donor-Derived Stromal Elements and HEVs Persist in Induced LNs

The relative contributions of donor and host-derived cell populations to the generation of the ectopic lymphoid structures were analyzed by making use of CD45 (Ly5)-congenic mice as well as β-actin GFP transgenic mice. The CD45.1 and CD45.2 mice were used to analyze the donor-derived hematopoietic cell populations that persist in the induced aggregates. Remarkably, 2 weeks postinduction, no donor-derived $CD45^+$ cells could be found in the induced lymphoid structures by immunohistochemistry ($n = 3$, data not shown), indicating that all hematopoietic cells that had lodged into the newly formed structure were host derived. To be able to determine the fate of both the hematopoietic and nonhematopoietic compartments from the injected newborn LNs, β-actin GFP mice were now utilized as the donor population. Two weeks after induction, the induced lymphoid structures were removed and analyzed by immunohistochemistry. Donor-derived cells in the induced lymphoid structure made up the lymphatic endothelium, identified by the expression of the hyaluronan receptor Lyve-1 (Jackson, 2003) (Figure 3A) and a population of stromal cells, which expressed VCAM-1 and ICAM-1 (Figures 3C and 3D). Adjacent to these stromal cells, the ERTR7 antigen, which has been described to be associated with the conduit system, was expressed, suggesting the establishment of a conduit system in these structures (Nolte et al., 2003) (Figure 3E). In addition, MAdCAM-1-expressing HEVs were of donor origin (Figure 3B). No CD11c-expressing dendritic cells of donor origin could be found, confirming our findings with the CD45-congenic mice (Figure 3F). Finally, analysis of B and T lymphocytes again showed that these cells were exclusively derived from the host (data not shown).

Induced Lymphoid Structures Support Recirculation of Naive Lymphocytes

The HEVs within the induced aggregates coexpressed the mucosal addressin MAdCAM-1 and the peripheral addressin PNAd. In order to determine whether these HEVs were functional, and whether the induced ectopic lymphoid structures had been integrated in the recirculation pathway of naïve lymphocytes, adult mice were given an intravenous injection of biotinylated spleno-

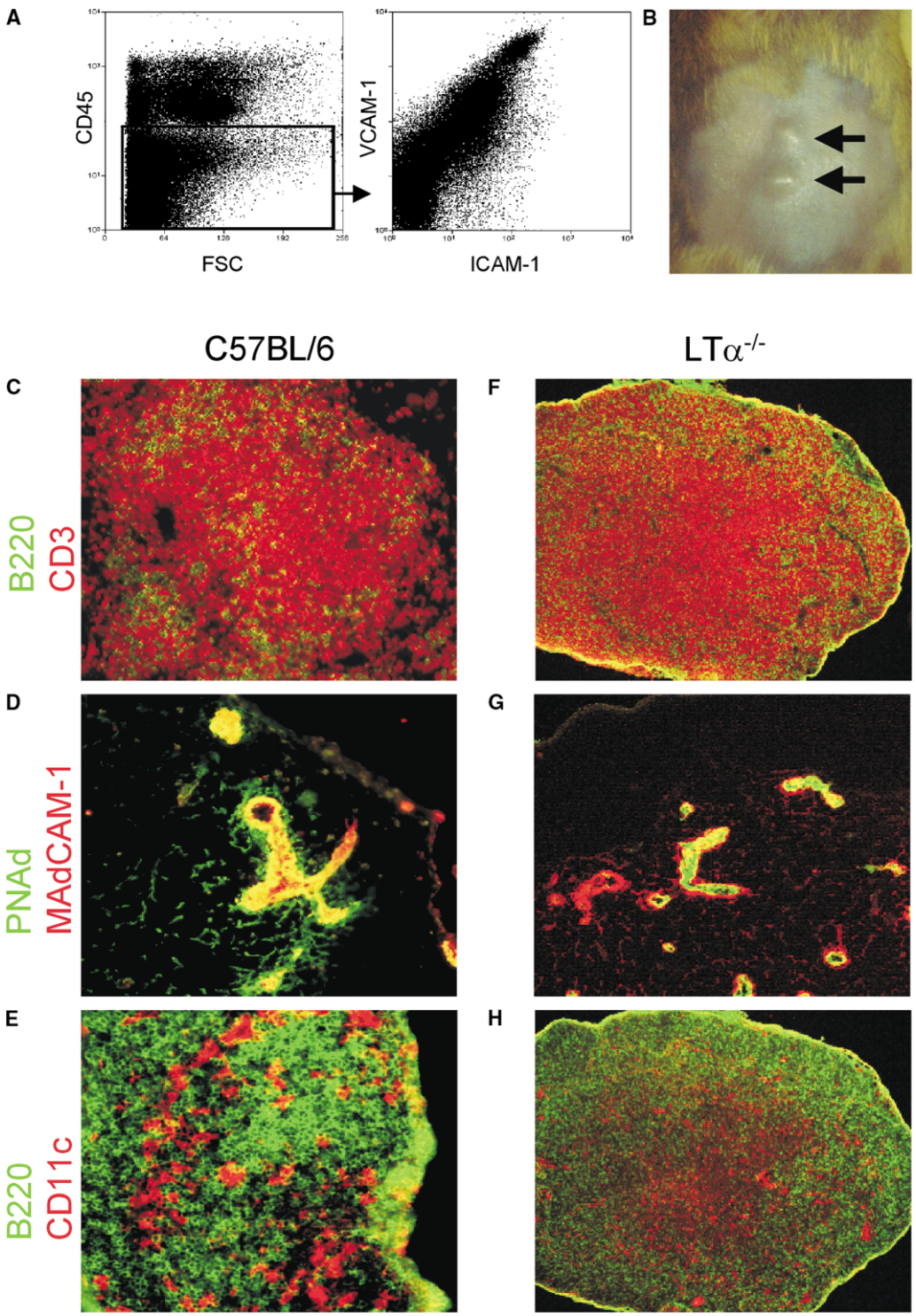

Figure 1. Induction of Lymphoid Neoorganogenesis in Adult C57BL/6 and LTα$^{-/-}$Mice

(A–H) Newborn C57BL/6 MLN cell suspensions were injected intradermally into the abdominal skin of either C57BL/6 or LTα$^{-/-}$ mice. Induced aggregates were dissected after 2 weeks. (A) The population of injected cells included CD45^{+} hematopoietic and CD45^{-} nonhematopoietic cells. The nonhematopoietic cells expresse ICAM-1 and VCAM-1. (B) Macroscopic image showing two lymphoid aggregates in the abdominal skin of a LTα$^{-/-}$ mouse. (C and F) B and T cells were present in the lymphoid aggregates induced by newborn LN cell injections in both (C) C57BL/6 and (F) LTα$^{-/-}$ mice (B cells are shown in green, and T cells are shown in red). (D and G) HEVs were present in the induced aggregates and expressed both MAdCAM-1 (red) and PNAd (green). (E and H) CD11c-positive dendritic cells (red) were also detected in both C57BL/6- and LTα$^{-/-}$-induced aggregates. Sections are representative for at least eight (LTα$^{-/-}$) to ten (C57BL/6) independent experiments.

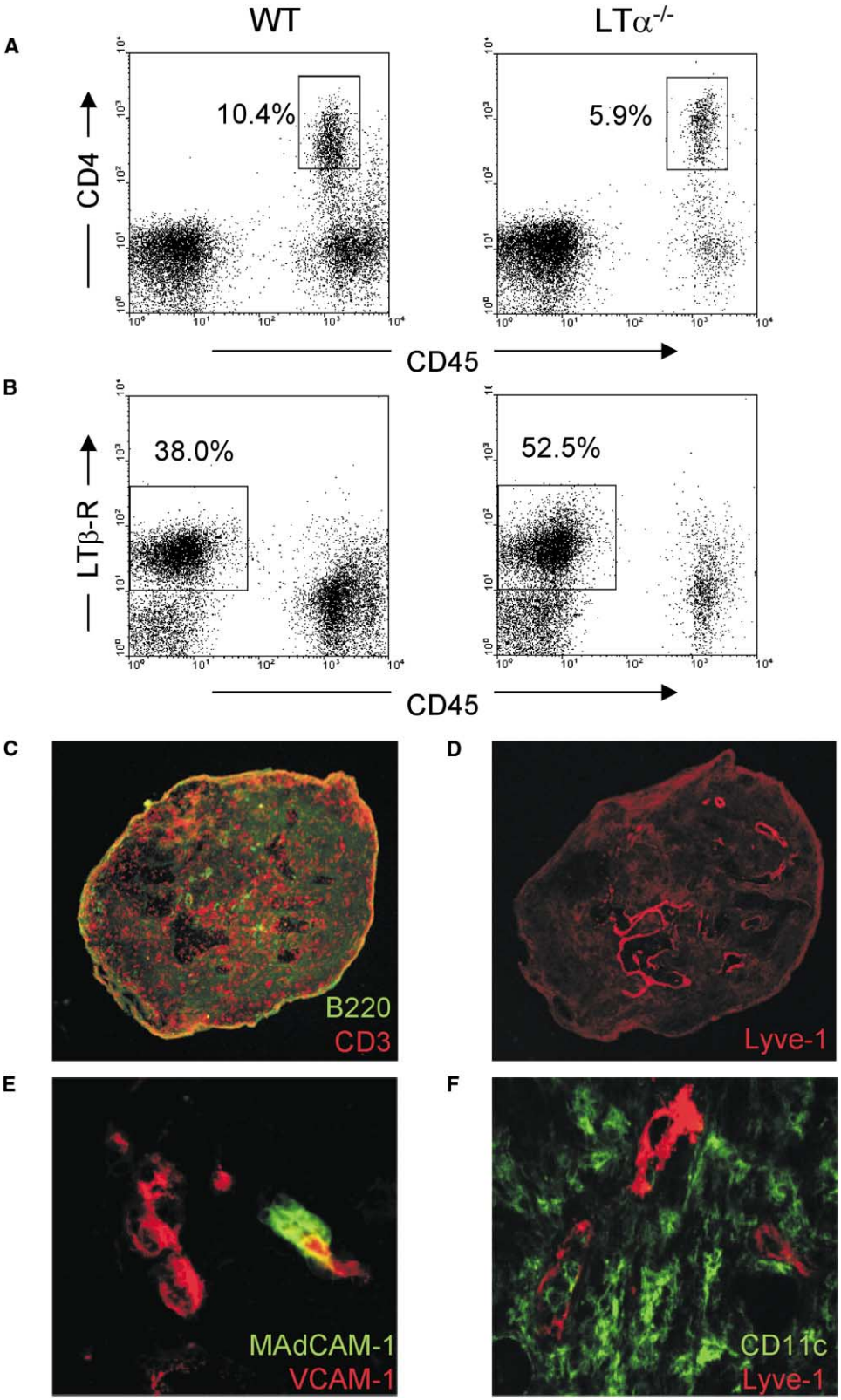

Figure 2. Induction of Lymphoid Structures Is Dependent on LTβ-R Signaling

Newborn rudimentary MLNs from $LT\alpha^{-/-}$ mice contain $CD45^+CD4^+CD3^-$ cells as well as $LT\beta\text{-}R^+$ stromal cells.

(A) The percentage of $CD45^+CD4^+CD3^-$ cells (absence of CD3 not shown) was reduced in $LT\alpha^{-/-}$ rMLN compared to C57BL/6 MLN.

(B) LTβ-R-expressing stromal cells were relatively more abundant in $LT\alpha^{-/-}$ rMLN. Equal numbers of cells for C57BL/6 and $LT\alpha^{-/-}$ are shown, and results are representative of three independent analyses.

(C–F) Coinjection of rMLN from newborn $LT\alpha^{-/-}$ mice with an agonistic anti-LTβ-R antibody was sufficient to induce the formation of lymphoid aggregates that contained (C) T cells (red) and few B cells (green), (D) Lyve-1-expressing lymphatic endothelium (red; B cells are shown in green), (E) MAdCAM-1- (green) and VCAM-1 (red)-expressing HEVs, as well as (F) $CD11c^+$ dendritic cells (green; Lyve-1 is shown in red).

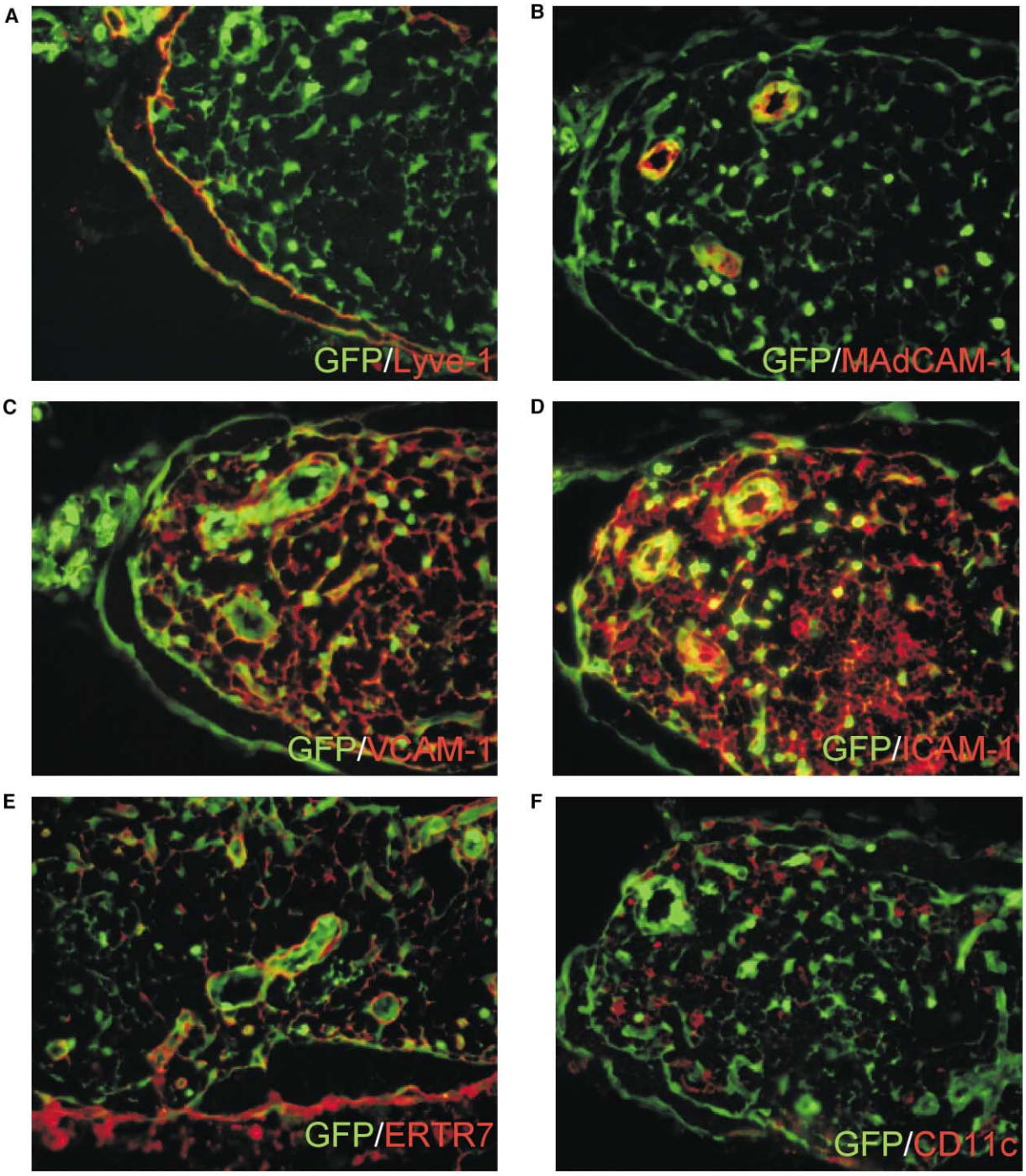

Figure 3. Donor Cells Persist in Induced Lymphoid Structures

Newborn MLN cells isolated from β-actin-GFP/C57BL/6 mice were injected into the abdominal skin of C57BL/6 mice. Induced aggregates were dissected after 2 weeks.

(A) The Lyve-1$^+$ lymphatic endothelium that surrounds the induced aggregate was of donor origin (Lyve-1 is shown in red; GFP is shown in green).

(B) MAdCAM-1-expressing HEVs were also of donor origin (MAdCAM-1 is shown in red; GFP is shown in green).

(C and D) The putative LN-organizing cells, which express (C) VCAM-1 and (D) ICAM-1, were also donor derived (ICAM-1 or VCAM-1 is shown in red; GFP is shown in green). The stromal cell marker ERTR7 was seen in close association with GFP$^+$ cells (ERTR-7 is shown in red; GFP is shown in green).

(E) CD11c$^+$ dendritic cells did not express the donor marker GFP (CD11c is shown in red; GFP is shown in green). Sections are representative of at least three independent experiments.

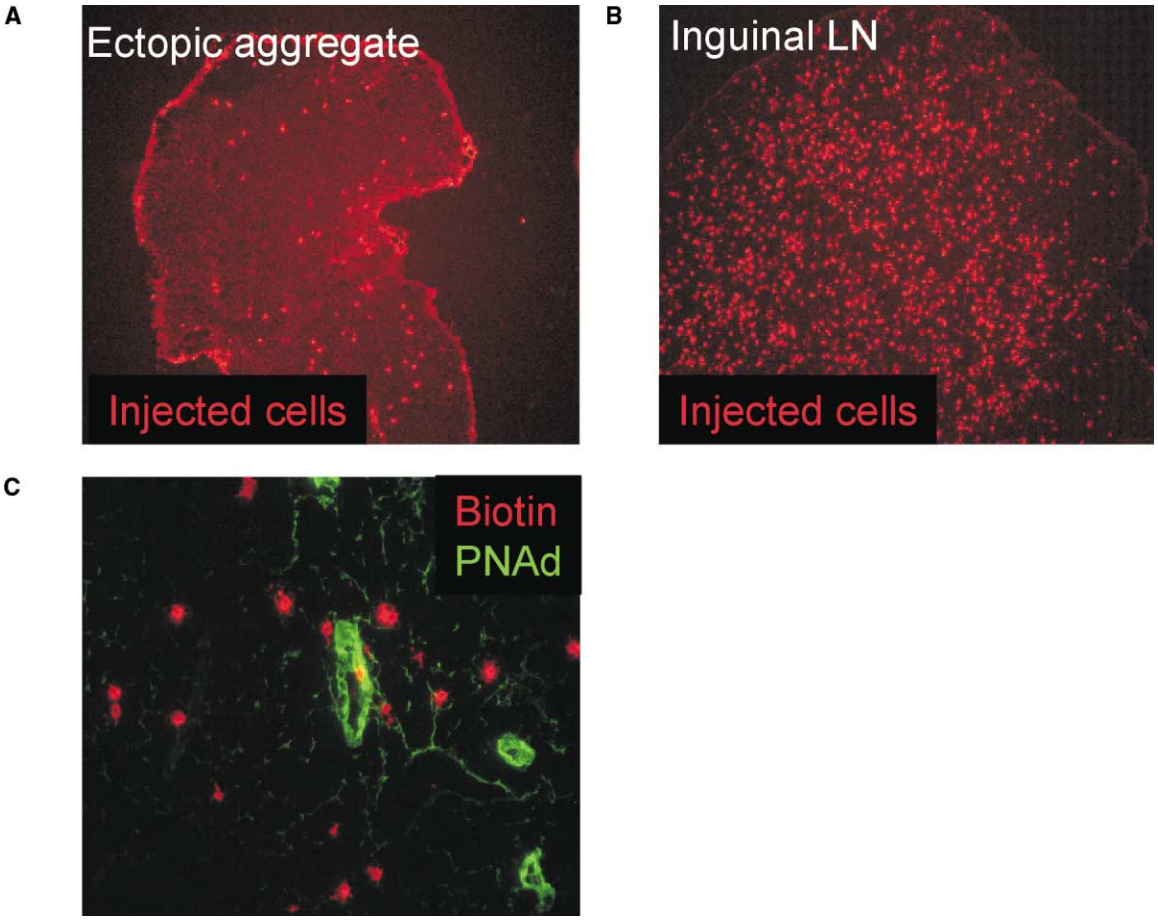

Figure 4. Induced Lymphoid Structures Support Recirculation of Naive Lymphocytes

Biotinylated splenocytes from C57BL/6 mice were adoptively transferred into adult C57BL/6 mice 2 weeks after induction of ectopic lymphoid aggregates.

(A and B) (A) Induced aggregates and (B) control inguinal LNs contained biotinylated cells 3 hr after injection.

(C) Transferred cells were seen entering the induced structure via the HEVs (PNAd is shown in green; injected cells are shown in red). Sections are representative of two independent experiments.

cytes 2 weeks after aggregate induction (Figure 4). After short-term (3 hr) migration, the induced lymphoid structures as well as control inguinal LNs were isolated, and upon immunohistochemical analysis, biotinylated cells were readily found in the inguinal LNs (Figure 4B), as well as in the induced ectopic aggregates (Figure 4A). Within the induced structures, cells could be visualized while transmigrating through the HEVs (Figure 4C). Although the number of cells detected in the ectopic structures was lower than in the control LNs, these data clearly show that a single injection of newborn LN-derived cells into the abdominal skin of adult mice induces not only the formation of an ectopic lymphoid aggregate, but also the local differentiation of blood vessels to support recirculation of naïve lymphocytes.

Induction of Lymphoid Neoorganogenesis in Neonatal Mice

The lack of distinctive lymphoid organ architecture within the induced lymphoid structures is a characteristic that is also seen in ectopic aggregates during chronic inflammation and may therefore reflect an intrinsic difference between the adult and early-postnatal immune system (Cupedo and Mebius, 2003; Takemura et al., 2001). In order to more closely mimic postnatal development, we set out to induce lymphoid structures in neonatal mice. Cells derived from newborn LNs were injected intradermally into the abdominal skin of mice at the day of birth, and after 2 weeks, LN-like structures could be dissected (Figure 5). In contrast to the ectopic aggregates induced in adult mice, immunohistochemical analysis of the aggregates induced in neonatal mice revealed a degree of organization reminiscent of normal LNs. T and B cells were separated into discrete areas, and anatomically distinct B cell follicles were now evident (Figure 5A). Furthermore, these follicles also contained FDCs (Figure 5B). The HEVs present in these structures still showed expression of both MAdCAM-1 and PNAd (data not shown). These data show that induction of lymphoid neoorganogenesis in neonatal mice results in the formation of an ectopic structure closely resembling LNs, rather than ectopic inflammatory aggregates.

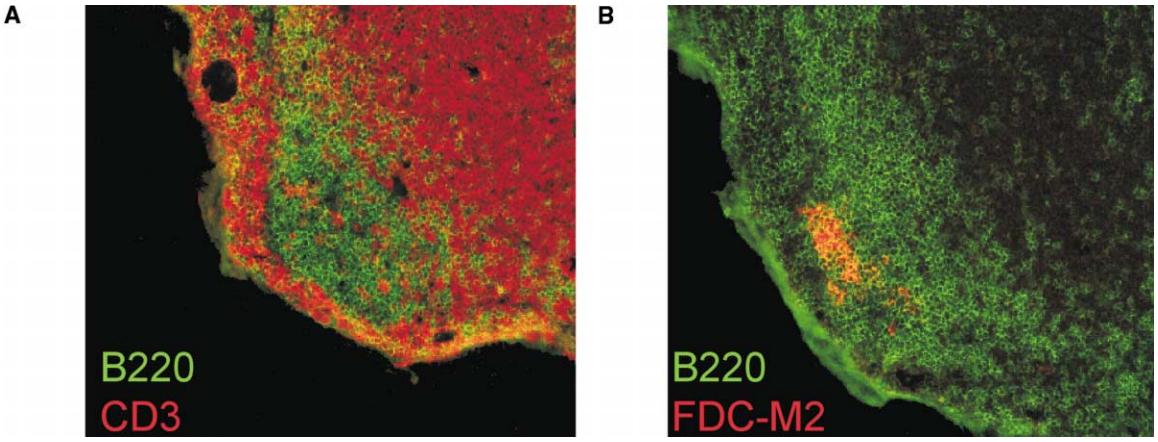

Figure 5. Induction of Organized LN-like Structures in Neonatal Mice

Newborn C57BL/6 MLN-derived cells were injected intradermally into the abdominal skin of C57BL/6 recipient mice at the day of birth and were analyzed after 2 weeks.

(A) T/B segregation and clearly distinct B cell follicles were present (B cells are shown in green; T cells are shown in red).

(B) The B cell follicles contained follicular dendritic cells, as identified by the FDC-M2 antibody (red). Sections are representative of at least ten independent experiments.

Divergent Roles for Host- versus Donor-Derived LT

During normal LN development, as well as during the formation of ectopic structures, LT is essential for the induction as well as the organization and maintenance of the lymphoid structure (Kang et al., 2002; Luther et al., 2000; Rennert et al., 1997, 1998). The fact that cells derived from C56BL/6 newborn LNs are capable of instigating lymphoid organogenesis in both C56BL/6 and LT$\alpha^{-/-}$ mice (Figure 1) indicated that for the initiation of LN development, LT expression by donor cells was sufficient. At birth, CD45^{+}CD4^{+}CD3^{-} cells are the major LT-expressing cell population and therefore likely the cells delivering the LT signal (Kim et al., 2000). To determine the contribution of donor-derived LT for subsequent organization of the induced aggregate, LT$\alpha^{-/-}$ mice were injected intradermally in the abdominal skin at the day of birth with cells from C56BL/6 neonatal LNs, and after 2 weeks, lymphoid structures were analyzed by immunohistochemistry. Strikingly, induction of lymphoid aggregates in newborn LT$\alpha^{-/-}$ mice led to the generation of structures phenotypically indistinguishable from the induced aggregates seen in adult C56BL/6 or LT$\alpha^{-/-}$ mice. T and B cells had not segregated into discrete areas (Figure 6A) and FDC development was absent (data not shown), while HEVs that coexpressed MAdCAM-1 and PNAd were again present (Figure 6B).

Further Organization in Ectopic Structures upon Immune Activation

To reproduce the sequence of events that is thought to lead to organized inflammatory infiltrates, we addressed whether ectopic structures that were generated in adult recipients could be organized through transient immune activation. Therefore, the abdominal skin surrounding the induced ectopic structure was exposed to the contact allergen oxazolone at 7 days after induction of the aggregate. Analysis 7 days later showed that although T/B cell segregation had increased and B cells, now located at the edge of the structure, were more tightly packed, FDC-containing B cell follicles could not be found (Figure 6C), while HEVs within these structures were well developed and expressed both MAdCAM-1 and PNAd (Figure 6D). Since FITC painted onto the abdominal skin reached the induced structures very inefficiently (data not shown), we assumed that transport of antigens from the skin to the ectopic structure via afferent lymphatics might not be optimal. Therefore, we chose to activate lymphocytes directly in vivo. Hereto, adult mice received an intradermal injection of neonatal MLN cells, and after 7 days, an agonistic anti-CD40 antibody was injected i.p. to mimic the upregulation of CD40L on activated T and NK cells. (Figures 6E–6H) (van Kooten and Banchereau, 2000). Injection of anti-CD40 will lead to the triggering of B cells, resulting in induction of LT and TNFα, as well as other cytokines, and will also directly effect dendritic cells to produce inflammatory cytokines and become fully mature (van Kooten and Banchereau, 2000; Worm and Geha, 1994). In addition, direct effects of anti-CD40 injections on FDCs or FDC precursors cannot be excluded. This would mimic the reported migration of activated T cells, which express CD40L, to the follicles (Ansel et al., 1999; Breitfeld et al., 2000; Sallusto et al., 1999; Schaerli et al., 2001; Schaerli et al., 2000). It has been suggested that, here, activated T cells might directly interact with FDCs (Liu et al., 1996). The induced structures were dissected after 14 days and were analyzed by immunofluoresence. In contrast to the disorganized structures normally found in adult animals, the mice that received anti-CD40 antibody now showed lymphoid aggregates with a higher degree of organization. T and B cells had segregated into separate areas (Figures 6E and 6G), with HEV expressing both PNAd and MADCAM-1 throughout the structure (Figure 6F), and FDCs present within B cell follicles (Figure 6H). This leads to the conclusion that, although donor-derived LT is sufficient to initiate the

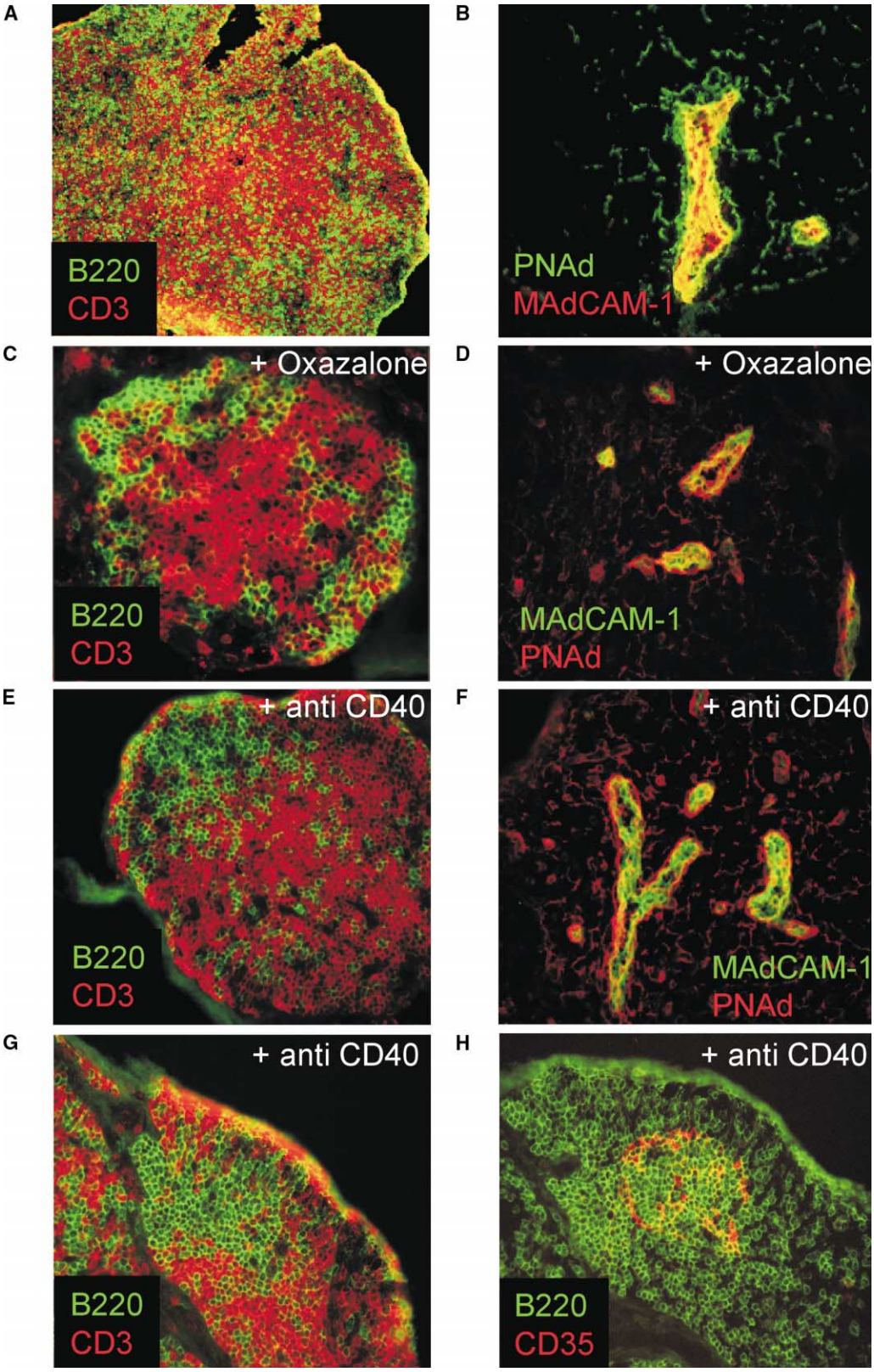

Figure 6. Requirements for Organization of the Induced Lymphoid Structures

Newborn C57BL/6 MLN cells were injected into the abdominal skin of LT$\alpha^{-/-}$ mice at the day of birth and were dissected after 2 weeks. (A) Within the induced aggregates, no clear T/B segregation could be seen (T cells are shown in red; B cells are shown in green). (B) In the absence of LTα expression in the recipient, both MAdCAM-1 (red) and PNAd (green) were still expressed on the HEVs. Sections are representative of at least seven independent experiments. To induce organization in ectopic aggregates formed in adult C57BL/6, mice

process of lymphoid neoorganogenesis, in adult mice, a subsequent immune activation, as mimicked by anti-CD40 injection, is needed to generate the cellular architecture typically associated with secondary lymphoid organs as well as organized inflammatory infiltrates.

FDCs Are Present in Induced Lymphoid Structures in TNF RI$^{-/-}$ Mice and Are Donor Derived

Differentiation of FDCs is dependent on combined signals from the LTβ-R and the TNF-RI, but the exact nature of the FDC precursor remains unclear (Endres et al., 1999; Fu et al., 1998). To address the issue of putative FDC precursors in neonatal LNs, newborn MLNs from C57BL/6 mice were used to induce lymphoid aggregates in TNF-RI$^{-/-}$ mice, which lack endogenous FDCs and B cell follicles (Pasparakis et al., 1997). Two weeks postintradermal injection, lymphoid structures were isolated and analyzed (Figures 7A and 7B). In contrast to the endogenous LNs found in TNF-RI$^{-/-}$ mice, the induced lymphoid structures displayed anatomically distinct B cell follicles (Figure 7A), which also contained FDCs (Figure 7B). This suggests that the neonatal LN contains TNF-RI-expressing cells that are capable of compensating for the absence of this receptor in TNF-RI$^{-/-}$ mice, and this compensation results in the normalization of the LN architecture.

To deliver absolute proof that FDC precursors are indeed present in neonatal LNs, neonatal MLNs from β-actin GFP mice were injected intradermally into neonatal C57BL/6 mice. Within the generated structures, FDC-containing B cell follicles were found (Figure 7C), and all FDCs were of donor origin (Figure 7D). Therefore, within the stromal compartment of neonatal LNs, precursors to FDCs are present.

Discussion

In this study, we show that a single intradermal injection of isolated newborn LN-derived cells leads to the induction of LN-like structures. These structures display all the characteristics of lymph nodes, i.e., they harbor B, T, and dendritic cells, functional HEVs, and lymphatic endothelium. Upon induction in neonatal mice, these ectopic structures are indistinguishable from normal lymph nodes, including FDC-containing B cell follicles located in the cortical regions, while T cells, dendritic cells, and functional HEVs are present in the interfollicular areas and more inward. The entire structure is encapsulated by lymphatic endothelial cells, and this architecture is analogous to the subcapsular sinus seen in lymph nodes. The donor-derived cells that persist within the newly formed LN-like structures are characterized by the expression of VCAM-1 and ICAM-1, and they therefore phenotypically resemble the organizing population in the developing PPs (Honda et al., 2001). Consequently, these cells also execute this organizing function in the ectopic structures by attracting and organizing the hematopoietic cells emigrating from the circulation. Interestingly, the injected suspension can reassemble into a stromal lymphoid framework and induce differentiation of functional blood vessels.

We show that the induction of ectopic LN-like structures depends on LT expression by donor-derived cells, while the organization within these structures requires LT expression by host cells. In the early postnatal murine immune system, CD45$^+$CD4$^+$CD3$^-$ cells are the most prevalent source of surface LT (Cupedo et al., 2002; Kim et al., 2000). With increasing age, the percentage of CD45$^+$CD4$^+$CD3$^-$ cells within the developing lymph nodes decreases from approximately 50% at birth to less then 0.1% in adult animals (Kim et al., 2000; Mebius et al., 1997). Induction of ectopic LN-like structures in neonatal mice will therefore likely lead to a situation in which the injected ICAM-1/VCAM-1$^+$-organizing cells in first instance interact with donor-derived CD45$^+$CD4$^+$ CD3$^-$ cells and subsequently attract host-derived CD45$^+$CD4$^+$ CD3$^-$ cells and some B cells (Luther et al., 2003). These recipient-derived cells are necessary for the completion of the lymphoid architecture by further providing required molecules such as LT, TNFα, and possibly other cytokines. Several days later, T cells start emigrating from the thymus and enter the developing organ via the HEVs, and this emigration mimics the sequence of events during normal development. The presence of high numbers of LT$^+$CD45$^+$CD4$^+$CD3$^-$ cells therefore seems a prerequisite for proper lymphoid organization. In the adult situation, the injected newborn LN-derived cells will attract mature rearranging lymphocytes, which fail to organize into discrete areas. This indicates that in the adult immune system a state of immune activation is required for the initiation of lymphoid organization, since, under pathological circumstances, organization into discrete T and B cell zones can be observed in tertiary lymphoid structures in patients (Hjelmstrom, 2001; Page et al., 2002; Salomonsson et al., 2002; Shi et al., 2001). Also, in mucosal lymphoid tissues, the presence of antigen can induce a higher level of organization. Immature isolated lymphoid follicles, which are clusters of B cells located in the wall of the intestine, become organized mature ILFs, containing germinal centers, upon luminal challenge by bacteria (Hamada et al., 2002; Lorenz et al., 2003). In our experiments, this situation of pathological immune activation was achieved by mimicking the presence of activated T cells through administration of anti-CD40. In vivo administration of anti-CD40 will lead to direct activation of B cells, resulting in the induction of LT and TNFα, as well as other cytokines (Worm and Geha, 1994). Furthermore, this may also directly stimulate dendritic cells and follicular dendritic

received either the contact allergen oxazolone painted onto the skin or 50 μg anti-CD40 antibody i.p. 1 week after LN induction.
(C) One week after oxazolone application, a more distinct separation of B and T cells could be observed, with B cells packed on the edge of the structure. However, FDC-containing B cell follicles were absent, while (D) HEV expressing both MAdCAM-1 (green) and PNAd (red) were present. (E and G) One week after anti-CD40 injection, induced aggregates contained distinct B cell areas (T cells are shown in red; B cells are shown in green), (F) while HEV expressing both MAdCAM-1 (green) and PNAd (red) were present.
(H) At times, FDC-M2$^+$ (data not shown) CD35$^+$ FDCs (FDCs are shown in red; B cells are shown in green) could be observed within B cell follicles. Sections are representative of three independent experiments.

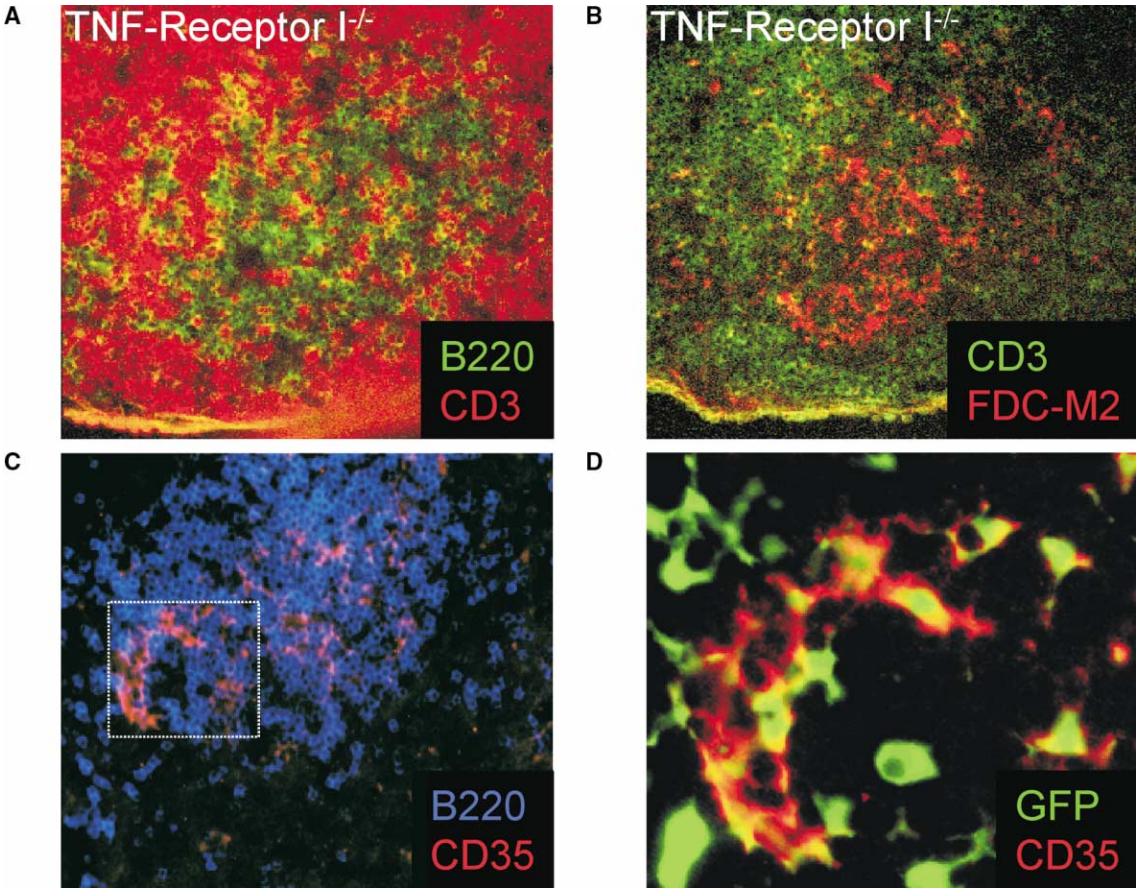

Figure 7. Origin of FDCs and Their Formation in TNF-RI$^{-/-}$ Mice

Ectopic aggregates were induced in newborn TNF-RI$^{-/-}$ mice.

(A) Two weeks postinduction in neonatal TNF-RI$^{-/-}$ mice, ectopic lymphoid aggregates had formed, complete with T/B segregation and distinct B cell follicles (B cells are shown in green; T cells are shown in red).

(B) In addition, B cell follicles in TNF-RI$^{-/-}$ mice contained follicular dendritic cells (T cells are shown in green; FDCs are shown in red). Sections are representative of two independent experiments. To determine FDC origin, newborn C57BL/6 mice were injected intradermally with β-actin GFP newborn MLN cells.

(C) After 2 weeks, FDC-containing B cell follicles were present (FDCs are shown in red; B cells are shown in blue).

(D) All FDCs were GFP^{+}, indicating their donor origin. Results are representative of three independent experiments.

cells. Triggering of all these subsets by anti-CD40 perfectly mimics the action of activated T cells, which transiently express CD40L upon activation (van Kooten and Banchereau, 2000). As a result, the formation of T and B cell areas, as well as FDCs, is initiated in the tertiary structures induced in adult mice. In the early neonatal immune system, this inducing signal is delivered by a specialized population of LT^{+}CD45^{+}CD4^{+}CD3^{-} cells, which are abundantly present at this time (Cupedo and Mebius, 2003).

The local differentiation of FDCs is dependent on signaling through LTβ-R as well as TNF-RI (Endres et al., 1999). In mice with a deletion of the gene encoding the TNF-RI, no FDCs develop, and, in addition, these mice show a disturbance of the LN architecture, with a lack of B cell follicles (Pasparakis et al., 1997). We show here that induction of ectopic lymphoid aggregates in neonatal TNF-RI$^{-/-}$ mice surprisingly leads to the generation of LN-like structures with FDC-containing B cell follicles that are indistinguishable from normal LNs. This suggests that within the newborn LN used for injection, TNF-RI-expressing cells are present that can develop into FDCs. Indeed, transfer of GFP-expressing donor cells now, for the first time to our knowledge, shows that within the neonatal LNs at the day of birth, FDC precursors are already present. This will now permit the identification of these precursors.

The model we describe here should allow for the definition of minimal cellular requirements for LN formation. However, we have not yet succeeded in establishing LN-like structures with sorted cellular subsets. Previously, transgenic expression of LTα, TNFα, CXCL13, or CCL21 was shown to be sufficient to induce various degrees of lymphoid neoorganogenesis (Fan et al., 2000; Kratz et al., 1996; Luther et al., 2000; Sacca et al., 1998), but the major disadvantage of these systems is the constitutive presence of the transgene, overruling any possible feedback mechanisms and leaving no room to determine the physiological induction of the transgenic cytokine. The model presented here does not have this disadvantage and more closely resembles the (patho-) physiological situation.

In sum, our data show that neonatal LNs contain all of the elements necessary to induce the novel formation of LN-like structures as well as tertiary lymphoid structures, two processes that we show to be strikingly similar. The degree of organization in secondary and tertiary structures depends on the presence of $CD45^+CD4^+CD3^-$ cells during development or activated lymphocytes under pathological conditions.

Experimental Procedures

Mice

C57BL/6 mice were purchased from Harlan and were kept under routine laboratory conditions. $LT\alpha^{-/-}$, TNF-$RI^{-/-}$, C57BL/6-Ly5.1, C57BL/6-Ly5.2, and β-actin-GFP/C57BL/6 mice (kindly provided by Dr. M. Okabe, Osaba University, Japan) were bred and kept at the in-house animal facilities of the VU Medical Center, Amsterdam.

Antibodies

For immunohistology and flow-cytometry, the following antibodies were used: 6B2 (anti-B220), KT3.1 (anti-CD3ϵ), GK-1.5 (anti-CD4), AL1-4A2 (anti-Ly5.2), MECA-367 (anti-MAdCAM-1), MECA-79 (anti-PNAd), MP33 (anti-CD45), 8C12 (anti-CD35/CR-1) (all were affinity purified from hybridoma cell culture supernatants with either protein G- or protein A-Sepharose [Pharmacia] and labeled in our laboratory when needed), FDC-M2 (anti-C4b, AMS Biotechnology, Ltd.), HL3 (anti-CD11c, Pharmingen), 429 (anti VCAM-1, Pharmingen), anti-ICAM-1 (Pharmingen), and anti-LTβ-R (Alexis Benelux, The Netherlands). Anti-Lyve-1 was kindly provided by D.G. Jackson, John Radcliffe Hospital, Oxford, United Kingdom, and the ERTR-7 antibody was a kind gift of P. Leenen, Erasmus Medical Center, Rotterdam, The Netherlands. LTβ-R-huIgG1 as well as control Fc fusion protein were obtained from Alexis.

Flow Cytometry

Mesenteric LNs were dissected at the day of birth, and cell suspensions were made by digestion with 0.5 mg/ml Collagenase type IV (Sigma) in PBS for 30 min at 37°C, while stirring continuously, and were subsequently filtered through a 100 μm nylon mesh. Flow cytometric analyses were performed on a FACS Calibur (Becton Dickinson).

Immunofluorescence Microscopy

Cryo-sections (6 μm) were fixed in dehydrated acetone for 5 min and air dried for an additional 10 min. Sections were incubated with primary antibody for 1 hr at room temperature, followed by a 30 min incubation with Fluor-Alexa-labeled conjugates (Molecular Probes) when needed. Sections were embedded in Fluorstab (ICN Biomedicals, Inc.) and analyzed on a Nikon Eclipse E800 microscope (Nikon Europe bv).

For visualization of GFP, aggregates were dissected and fixed for 3 hr in 4% (v/v) formaline in PBS, followed by an overnight equilibration in a 20% (w/v) sucrose solution in PBS.

Lymph Node Induction

Mesenteric LNs were dissected at the day of birth, and cell suspensions were made by digestion with 0.5 mg/ml Collagenase type IV (Sigma) in PBS for 30 min at 37°C, while stirring continuously, and were subsequently filtered through a 100 μm nylon mesh. A total of 2–3 $\times$ 10^5 newborn LN cells (equivalent to approximately 2 day 0 MLNs) were resuspended in 5 or 10 μl PBS and injected intradermally into the abdominal skin of either newborn or adult recipients, respectively.

For induction of organization after aggregate induction in adult animals, either oxazolone (4-ethoxyethylene-2-phenyl-oxazol-5-one; Sigma) (100 μl of a 10% solution in a 4:1 acetone:olive oil mixture) was painted onto the skin or 50 μg 1C10 (anti-CD40, a kind gift of Dr. J. de Haan, VUMC, Amsterdam) was injected i.p. 7 days after induction. Aggregates were dissected 1 week later.

For inductions with $LT\alpha^{-/-}$ cells, 10–20 $\times$ 10^5 cells from $LT\alpha^{-/-}$ rudimentary MLNs were coinjected with an agonistic anti-LTβ-R antibody (1 μg).

Recirculation

Spleens were isolated from C57BL/6 mice, and erythrocytes were removed by alkaline lysis. Splenocytes were biotinylated by incubation in 80 μg/ml Biotin-7-NHS in PBS (Boerhringer, Mannheim, Germany) for 15 min at room temperature while shaking continuously. After washing, 2–5 $\times$ 10^7 biotinylated cells were injected intravenously in mice 2 weeks postinduction of aggregates. Recipient mice were sacrificed after 3 hr, and induced aggregates as well as control inguinal LNs were harvested.

Acknowledgments

We would like to thank the staff of the animal facilities at the VU Medical center. T.C. was supported by the Netherlands Organization for Scientific Research (grant #901-06-340).

Received: January 30, 2004
Revised: August 6, 2004
Accepted: August 8, 2004
Published: November 16, 2004

References

Amft, N., Curnow, S.J., Scheel-Toellner, D., Devadas, A., Oates, J., Crocker, J., Hamburger, J., Ainsworth, J., Mathews, J., Salmon, M., et al. (2001). Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjogren's syndrome. Arthritis Rheum. *44*, 2633–2641.

Ansel, K.M., McHeyzer-Williams, L.J., Ngo, V.N., McHeyzer-Williams, M.G., and Cyster, J.G. (1999). In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J. Exp. Med. *190*, 1123–1134.

Ansel, K.M., Ngo, V.N., Hyman, P.L., Luther, S.A., Förster, R., Sedgwick, J., Browning, J.L., Lipp, M., and Cyster, J.G. (2000). A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature *406*, 309–314.

Armengol, M.P., Juan, M., Lucas-Martin, A., Fernandez-Figueras, M.T., Jaraquemada, D., Gallart, T., and Pujol-Borrell, R. (2001). Thyroid autoimmune disease: demonstration of thyroid antigen-specific B cells and recombination-activating gene expression in chemokine-containing active intrathyroidal germinal centers. Am. J. Pathol. *159*, 861–873.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., and Forster, R. (2000). Follicular B helper T cells express CXC chemokine receptor *5*, localize to B cell follicles, and support immunoglobulin production. J. Exp. Med. *192*, 1545–1552.

Cuff, C.A., Schwartz, J., Bergman, C.M., Russell, K.S., Bender, J.R., and Ruddle, N.H. (1998). Lymphotoxin alpha3 induces chemokines and adhesion molecules: insight into the role of LT alpha in inflammation and lymphoid organ development. J. Immunol. *161*, 6853–6860.

Cupedo, T., and Mebius, R.E. (2003). Role of chemokines in the development of secondary and tertiary lymphoid tissues. Semin. Immunol. *15*, 243–248.

Cupedo, T., Kraal, G., and Mebius, R.E. (2002). The role of CD45+CD4+CD3− cells in lymphoid organ development. Immunol. Rev. *189*, 41–50.

Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.-W., Karin, M., Ware, C.F., and Green, D.R. (2002). The lymphotoxin-β receptor induces different patterns of gene expression via two NF-κB pathways. Immunity *17*, 525–535.

Eberl, G., Marmon, S., Sunshine, M.J., Rennert, P.D., Choi, Y., and Littman, D.R. (2004). An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat. Immunol. *5*, 64–73.

Endres, R., Alimzhanov, M.B., Plitz, T., Fütterer, A., Kosco-Vilbois, M.H., Nedospasov, S.A., Rajewsky, K., and Pfeffer, K. (1999). Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lympho-

toxin beta and tumor necrosis factor by B cells. J. Exp. Med. *189*, 159–168.

Fan, L., Reilly, C.R., Luo, Y., Dorf, M.E., and Lo, D. (2000). Cutting edge: ectopic expression of the chemokine TCA4/SLC is sufficient to trigger lymphoid neogenesis. J. Immunol. *164*, 3955–3959.

Finke, D., Acha-Orbea, H., Mattis, A., Lipp, M., and Kraehenbuhl, J. (2002). $CD4^+CD3^-$ cells induce Peyer's patch development. Role of $\alpha4\beta1$ integrin activation by CXCR5. Immunity *17*, 363–373.

Fu, Y.X., and Chaplin, D.D. (1999). Development and maturation of secondary lymphoid tissues. Annu. Rev. Immunol. *17*, 399–433.

Fu, Y.-X., Huang, G., Matsumoto, M., Molina, H., and Chaplin, D.D. (1997). Independent signals regulate development of primary and secondary follicle structure in spleen and mesenteric lymph node. Proc. Natl. Acad. Sci. USA *94*, 5739–5743.

Fu, Y.X., Huang, G., Wang, Y., and Chaplin, D.D. (1998). B lymphocytes induce the formation of follicular dendritic cell clusters in a lymphotoxin alpha-dependent fashion. J. Exp. Med. *187*, 1009–1018.

Fukuyama, S., Hiroi, T., Yokota, Y., Rennert, P.D., Yanagita, M., Kinoshita, N., Terawaki, S., Shikina, T., Yamamoto, M., Kurono, Y., et al. (2002). Initiation of NALT organogenesis is independent of the IL-7R, LTbetaR, and NIK signaling pathways but requires the Id2 gene and CD3(−)CD4(+)CD45(+) cells. Immunity *17*, 31–40.

Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H., and Pfeffer, K. (1998). The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity *9*, 59–70.

Hamada, H., Hiroi, T., Nishiyama, Y., Takahashi, H., Masunaga, Y., Hachimura, S., Kaminogawa, S., Takahashi-Iwanaga, H., Iwanaga, T., Kiyono, H., et al. (2002). Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. J. Immunol. *168*, 57–64.

Han, S., Cao, S., Bheekha-Escura, R., and Zheng, B. (2001). Germinal center reaction in the joints of mice with collagen-induced arthritis: an animal model of lymphocyte activation and differentiation in arthritis joints. Arthritis Rheum. *44*, 1438–1443.

Hashi, H., Yoshida, H., Honda, K., Fraser, S., Kubo, H., Awane, M., Takabayashi, A., Nakano, H., Yamaoka, Y., and Nishikawa, S. (2001). Compartmentalization of Peyer's patch anlagen before lymphocyte entry. J. Immunol. *166*, 3702–3709.

Hjelmstrom, P. (2001). Lymphoid neogenesis: de novo formation of lymphoid tissue in chronic inflammation through expression of homing chemokines. J. Leukoc. Biol. *69*, 331–339.

Honda, K., Nakano, H., Yoshida, H., Nishikawa, S., Rennert, P., Ikuta, K., Tamechika, M., Yamaguchi, K., Fukumoto, T., Chiba, T., et al. (2001). Molecular basis for hematopoietic/mesenchymal interaction during initiation of peyer's patch organogenesis. J. Exp. Med. *193*, 621–630.

Jackson, D.G. (2003). The lymphatics revisited: new perspectives from the hyaluronan receptor LYVE-1. Trends Cardiovasc. Med. *13*, 1–7.

Kang, Y.M., Zhang, X., Wagner, U.G., Yang, H., Beckenbaugh, R.D., Kurtin, P.J., Goronzy, J.J., and Weyand, C.M. (2002). CD8 T cells are required for the formation of ectopic germinal centers in rheumatoid synovitis. J. Exp. Med. *195*, 1325–1336.

Kim, D., Mebius, R.E., MacMicking, J.D., Jung, S., Cupedo, T., Castellanos, Y., Rho, J., Wong, B.R., Josien, R., Kim, N., et al. (2000). Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. J. Exp. Med. *192*, 1467–1478.

Kratz, A., Campos-Neto, A., Hanson, M.S., and Ruddle, N.H. (1996). Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. J. Exp. Med. *183*, 1461–1472.

Liu, Y.J., Grouard, G., de Bouteiller, O., and Banchereau, J. (1996). Follicular dendritic cells and germinal centers. Int. Rev. Cytol. *166*, 139–179.

Lorenz, R.G., Chaplin, D.D., McDonald, K.G., McDonough, J.S., and Newberry, R.D. (2003). Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. J. Immunol. *170*, 5475–5482.

Luther, S.A., Ansel, K.M., and Cyster, J.G. (2003). Overlapping roles of CXCL13, interleukin 7 receptor alpha, and CCR7 ligands in lymph node development. J. Exp. Med. *197*, 1191–1198.

Luther, S.A., Lopez, T., Bai, W., Hanahan, D., and Cyster, J.G. (2000). BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. Immunity *12*, 471–481.

Mebius, R.E. (2003). Organogenesis of lymphoid tissues. Nat. Rev. Immunol. *3*, 292–303.

Mebius, R.E., Rennert, P., and Weissman, I.L. (1997). Developing lymph nodes collect $CD4^+CD3^-$ $LTbeta^+$ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. Immunity *7*, 493–504.

Muller, J.R., and Siebenlist, U. (2003). Lymphotoxin beta receptor induces sequential activation of distinct NF-kappa B factors via separate signaling pathways. J. Biol. Chem. *278*, 12006–12012.

Ngo, V.N., Korner, H., Gunn, M.D., Schmidt, K.N., Riminton, D.S., Cooper, M.D., Browning, J.L., Sedgwick, J.D., and Cyster, J.G. (1999). Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. J. Exp. Med. *189*, 403–412.

Ngo, V.N., Cornall, R.J., and Cyster, J.G. (2001). Splenic T zone development is B cell dependent. J. Exp. Med. *194*, 1649–1660.

Nolte, M.A., Belien, J.A., Schadee-Eestermans, I., Jansen, W., Unger, W.W., van Rooijen, N., Kraal, G., and Mebius, R.E. (2003). A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. J. Exp. Med. *198*, 505–512.

Page, G., Lebecque, S., and Miossec, P. (2002). Anatomic localization of immature and mature dendritic cells in an ectopic lymphoid organ: correlation with selective chemokine expression in rheumatoid synovium. J. Immunol. *168*, 5333–5341.

Pasparakis, M., Alexopoulou, L., Grell, M., Pfizenmaier, K., Bluethmann, H., and Kollias, G. (1997). Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor. Proc. Natl. Acad. Sci. USA *94*, 6319–6323.

Rennert, P.D., Browning, J.L., Mebius, R., Mackay, F., and Hochman, P.S. (1996). Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. J. Exp. Med. *184*, 1999–2006.

Rennert, P.D., Browning, J.L., and Hochman, P.S. (1997). Selective disruption of lymphotoxin ligands reveals a novel set of mucosal lymph nodes and unique effects on lymph node cellular organization. Int. Immunol. *9*, 1627–1639.

Rennert, P.D., James, D., Mackay, F., Browning, J.L., and Hochman, P.S. (1998). Lymph node genesis is induced by signaling through the lymphotoxin beta receptor. Immunity *9*, 71–79.

Sacca, R., Cuff, C.A., Lesslauer, W., and Ruddle, N.H. (1998). Differential activities of secreted lymphotoxin-alpha3 and membrane lymphotoxin-alpha1beta2 in lymphotoxin-induced inflammation: critical role of TNF receptor 1 signaling. J. Immunol. *160*, 485–491.

Sallusto, F., Kremmer, E., Palermo, B., Hoy, A., Ponath, P., Qin, S., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. Eur. J. Immunol. *29*, 2037–2045.

Salomonsson, S., Larsson, P., Tengner, P., Mellquist, E., Hjelmstrom, P., and Wahren-Herlenius, M. (2002). Expression of the B cell-attracting chemokine CXCL13 in the target organ and autoantibody production in ectopic lymphoid tissue in the chronic inflammatory disease Sjogren's syndrome. Scand. J. Immunol. *55*, 336–342.

Schaerli, P., Willimann, K., Lang, A.B., Lipp, M., Loetscher, P., and Moser, B. (2000). CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J. Exp. Med. *192*, 1553–1562.

Schaerli, P., Loetscher, P., and Moser, B. (2001). Cutting edge: induction of follicular homing precedes effector Th cell development. J. Immunol. *167*, 6082–6086.

Shi, K., Hayashida, K., Kaneko, M., Hashimoto, J., Tomita, T., Lipsky, P.E., Yoshikawa, H., and Ochi, T. (2001). Lymphoid chemokine B cell-

attracting chemokine-1 (CXCL13) is expressed in germinal center of ectopic lymphoid follicles within the synovium of chronic arthritis patients. J. Immunol. *166*, 650–655.

Takemura, S., Braun, A., Crowson, C., Kurtin, P.J., Cofield, R.H., O'Fallon, W.M., Goronzy, J.J., and Weyand, C.M. (2001). Lymphoid neogenesis in rheumatoid synovitis. J. Immunol. *167*, 1072–1080.

van Kooten, C., and Banchereau, J. (2000). CD40–CD40 ligand. J. Leukoc. Biol. *67*, 2–17.

Weyand, C.M., Kurtin, P.J., and Goronzy, J.J. (2001). Ectopic lymphoid organogenesis: a fast track for autoimmunity. Am. J. Pathol. *159*, 787–793.

Worm, M., and Geha, R.S. (1994). CD40 ligation induces lymphotoxin alpha gene expression in human B cells. Int. Immunol. *6*, 1883–1890.